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ANALYZING AND COMMUNICATING RELATIVE ANTIBODY ACTIVITY AS MEASURED BY ENZYME IMMUNOASSAY (EIA)

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Due to the rapid advances in enzyme immunoassay technology, there is a need for carefully considering the means by which laboratory results are reported to the clinician. The problem is especially acute with the enzyme-linked immunosorbent assay (ELISA) for measuring specific antibody activity. For routine diagnostic applications, the meaning of the assay result is of paramount importance, for no matter how sensitive and accurate the technique, the result has little value unless it is in a form intelligible to the user, the clinician.

Comparative Problems of Reporting Antigen Antibody Concentrations by Enzyme Immunoassay

The end results of quantitative enzyme immunoassay of antigen or hapten concentration, as determined by the standard curve method, is self evident because the result is expressed in absolute terms, µg/ml for example (Figure 1).

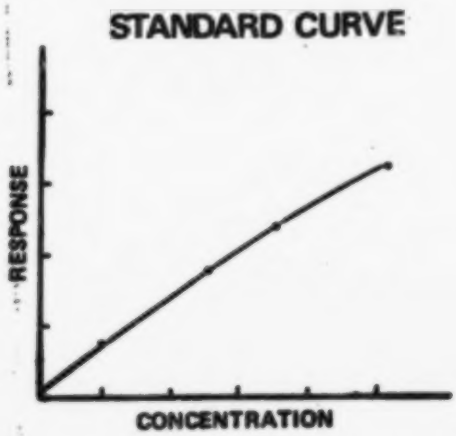

Figure 1. Standard curve for a competitive equilibrium reaction. A measure response (Y-axis) is directly correlatable to its corresponding concentration (X-axis).

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This is an ideal report for clinical purposes, especially when accompanied by the range of normal or pathological values. The use of the standard curve (dose-response curve) method in antigen assays is made possible by certain fundamental features of such assays:

1. The concentration range of the substance assayed is known and rarely spans more than two $\log_{10}$ dilutions, often less than one $\log_{10}$ dilution. For example, digoxin, a cardioactive drug used in the treatment of chronic heart disease, never exceeds 10 ng/ml. Sensitive assays can be designed for narrow concentration ranges. As the range increases, sensitivity usually decreases.
2. In antigen assays we only have to deal with the affinity constant of a single antibody preparation, that directed against the antigen. Therefore, a single standard curve can be prepared and conditions of the test adjusted so that the required concentration range falls largely within that part of the standard curve having maximum slope and hence, maximum sensitivity. For greater accuracy, this dose-response curve can be transformed to a linear approximation using a choice of mathematical transforms such as logit-log, spline-fit, third order polynomial, or four parameter logistic curve fit. The latest generation of automated and semi-automated ELISA microplate readers have designed-in a capability to accept programmable calculators which are able to compute standard curve parameters and transform ELISA values into concentration units for print-out. The vast experience with standard curve-fitting gained from radioimmunoassay applications are largely applicable to EIA for quantitation of antigen.

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In contrast to antigen quantitation, the measurement of antibody by EIA is more difficult. The concentration range of specific antibody in pathological sera can span up to six $\log_{10}$ dilutions (Figure 2).

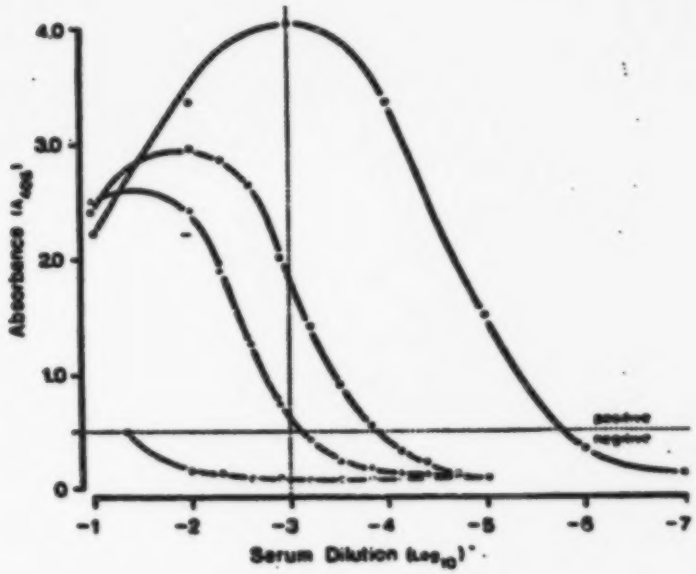

Figure 2. Indirect ELISA serum dose-response curves for human IgG anti-Toxocara antibody, illustrating the range of antibody activity that can occur. Intercepts with the vertical line indicate the absorbance values at working serum dilution (10^{-3}). Intercepts with the horizontal line indicate titer.

(●) Strong Positive :Titer = 1:800,000 A_{405} = 4.0
(■) Moderate Positive :Titer = 1:8,000 A_{405} = 1.80
(▲) Weak Positive :Titer = 1:1,100 A_{405} = 0.55
(○) Negative :Titer = 1:30 A_{405} = 0.09

This figure illustrates several dose-response curves for separate antisera containing anti-Toxocara antibody. These results point out the problem that must be dealt with in an EIA which tests only one serum dilution (10^{-3} in this case) when a wide range of antibody activity is possible. Positive/negative cut off was found to be an absorbance of 0.50. When all sera were assayed at this dilution, the weak positive serum ( ) having only an absorbance of 0.55 at this dilution nearly was incorrectly classified as negative. This vividly illustrates the difficulty in designing an assay to cover this broad range of concentration.

The second problem associated with measuring antibody concentration by the standard curve method arises from the fact that antibody affinities of different sera, even from the same patient, cannot be assumed to be identical. And in fact, this is the assumption we make when we interpolate a concentration corresponding to a given dose for one antibody from a standard curve prepared with a second antibody preparation. Why is this a mistake? Observed response is based upon the amount of antibody bound to the antigen. From the Law of Mass Action we see from the expression

$$[Ag] + [Ab] \xrightarrow{Ka} [AgAb]$$

that the amount of Ab bound to the antigen at any given time is dependent on two parameters: (1) the concentration of the reactants, [Ag] and [Ab], and (2) the affinity of the antibody for the antigen as expressed by the affinity constant, Ka. Therefore, titer can best be described by the expression:

$$\text{Titer} = Ka[Ab]$$

From this relationship you can see that the amount of Ab bound in an EIA is not totally determined by the concentration of Ab present. It is possible for a sera containing high Ab levels with a low Ka to give a response less than a lower concentration of more avid antibody. Therefore, standard curves prepared from equal Ab concentration (ng/IgG ml) can have quite different appearances and response values on these curves can correspond to very different antibody concentrations.

To further complicate the problem, in tests for a specific antibody response of a particular immunoglobulin class, the dose-response curve can be altered by the presence or absence of response in other immunoglobulin classes (Figure 3).

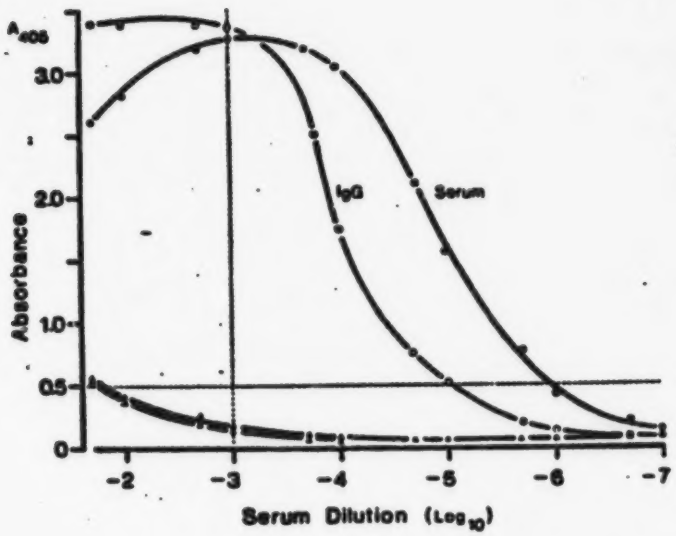

Figure 3. IgG anti-Toxocara antibody dose-response curves for positive (●) and negative (▲) sera and for the same sera (○, △) following the removal of IgM by fractionation on Bio-Gel A-5m. The absorbances at the serum working dilution (10^{-3}) remain unaltered, however the slope of the positive dose-response curve increases to produce a ten-fold drop in titer. The prozone phenomenon is also diminished.

This group of standard curves is based upon results of EIA's of the same two sera, a positive and a negative, with the IgM present and after it had been removed. Removal of the IgM had no effect on the negative serum test results as expected. For the positive serum, removal of the IgM did not alter the absorbance at the working dilution (10^{-3}). However, the slope of the standard curve increases to produce a ten-fold drop in titer. The prozone phenomenon is also diminished.

Therefore, antibody affinity and the response of more than one antibody class make several dose-response curves possible even in the same patient. For these and other reasons mentioned here the standard curve method has rarely

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been used to produce results in absolute units; for example, specific antibody level = 70 ng/IgG ml. A variety of alternative approaches has evolved to communicate an estimate of relative "antibody activity" rather than absolute concentration. Some are borrowed from traditional serology, while others are unique contributions, but none is entirely satisfactory.

What are the ideal requirements of EIA serological reports? No serological test can be both 100% specific and 100% sensitive, but it is still reasonable to list ideal characteristics desirable in the presentation of a test result to the clinician.

1. The ideal report must be easily understood by the clinician who will not necessarily be familiar with the laboratory details of ELISA techniques and data products.
2. The report should provide some qualitative information regarding the status of the patient; for example, whether the specific antibody activity is "Positive" or "Negative" or, more realistically, "Reactive" or "Non-reactive" or above or within the "normal" range.
3. The logical extension of the "positive" qualitative report is a quantitative report which will provide an index of the severity or recency of infection and will allow serial follow-up of the patient and comparison to other patients.
4. To be ideally quantitative, a scale is needed that is linearly proportional to antibody activity, numerical, continuous (i.e., step-less) and consistent in its unitage and significance.
5. It would be useful if the report is comparative such that it presents a measure of the patient's antibody activity relative to that found in a reference normal or pathological group.

6. Ideally, the basis by which the test data are processed to a clinical report should not include assumptions; such as assuming a normal or gaussian distribution of response values, or assumptions of parallelism of dose-response curves.
7. Results generated by the ideal method should, in their final form, be adequate for retrospective and prospective sero-epidemiologic analysis. A concentration value from today's assay must relate to past and future values.
8. To optimize the efficiency and economy of EIA as a proper assay, the report should be based on tests at a single serum dilution.

Setting the Positive/Negative Discrimination Level

One of the first and most difficult tasks in measuring antibody concentrations, whether the test is qualitative or quantitative, is discriminating between positive and negative. Various methods for determining the "positive/negative cut-off" line have been used but the common method is to set the minimum positive response value (e.g., Absorbance) at 2 or 3 times the mean absorbance value of the negative group. This method is used only when small numbers of reference normal sera (<10) are available and is not recommended because of inadequate data.

When more reference sera (10-100) are available, the minimum positive absorbance is set at the mean negative value plus two or three standard deviations (Figure 4).

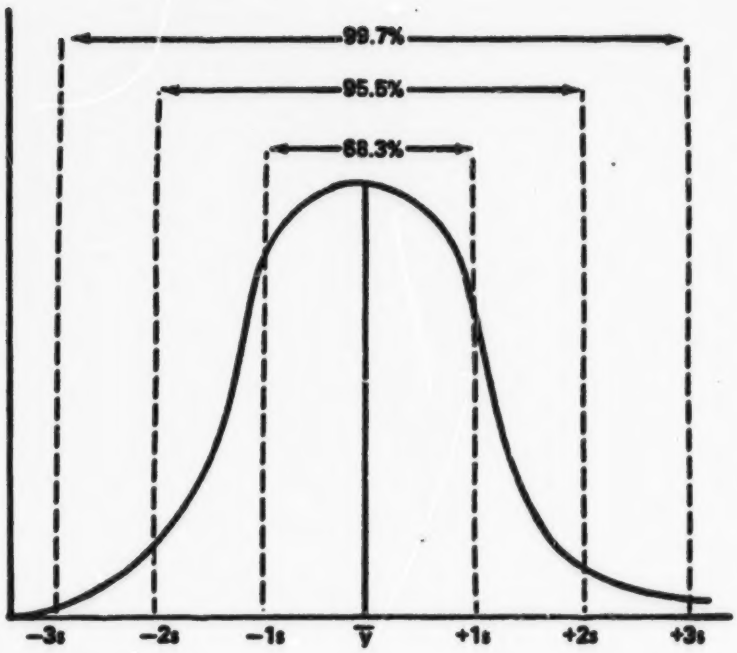

Figure 4. Gaussian distribution.

These limits state that only 1 observation in 20 or 1 observation in 100 respectively, will exceed these limits based upon chance alone. This method is rarely valid because it assumes that EIA values in the normal population are normally distributed while they, along with most other measures of serum constituents in normal populations, are distributed with a positive skew (Figure 5).

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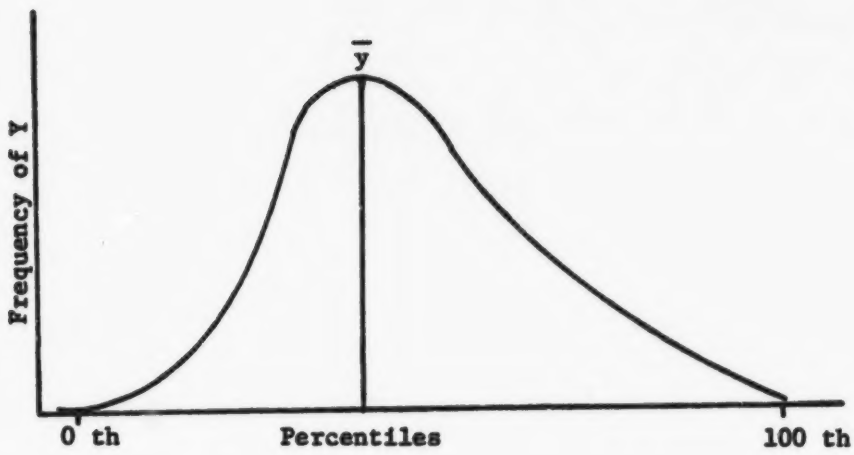

Figure 5. A distribution skewed in the positive direction.

A normal distribution assumes that both tails of the curve (area below -2s and above +2s, Figure 4) contain the same number of observations. If the distribution is actually skewed in the positive direction (Figure 5) then the upper tail (+2s) will contain more observations than expected. Therefore, type 1 errors (false positives) occur at +2 and +3 standard deviations.

Where absorbance values are not normally distributed, non-parametric methods should be used. The upper limit can then be determined from a minimum of 120 sera from the reference normal group and set at the 97.5th or more conservatively, the 100th percentile. The value corresponding to the 97.5th percentile may be calculated by the procedure:

$$r = \frac{\text{Percentile } (n + 1)}{100}$$

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For a 120 determination sample, then, the r^{th} value (the 2.5 percentile):

$$r = \frac{2.5\ (120 + 1)}{100} = 3.025$$

The 2.5 percentile then is the 3.025th value from the low end. In other words, the value that corresponds to 2.5% of the difference between the 3rd and 4th values from that end.

Let us suppose that the 5 lowest values that we have found in a sample of 120 determinations of serum total thyroxine are 3.9, 4.2, 4.3, 4.5, 4.9. Then, the 3.025th value is that one corresponding to 2.5% of the difference between the 3rd and 4th values. In this example it would be the value located at 2.5% of the distance between 4.3 and 4.5. This distance being 0.2, then, 2.5 of 0.2 = 0.005, which we add to the third value:

$$4.3 + 0.005 = 4.305$$

This is our lower limit. We would round off to 4.3.

Likewise, the 97.5th percentile would be the 3.025th value from the high end, or the value corresponding to 2.5% of the difference between the 3rd and 4th values from that end. The 5 highest values for our 120 samples are 14.1, 14.2, 14.7, 14.8, 14.9. Then the 3.025th value is that one corresponding to 2.5% of the difference between the 3rd and 4th values of this end of the sample. In this case, 2.5% of the distance between 14.7 and 14.2. Or 0.5 X 0.025 = 0.0125. This would be subtracted from the 3rd value, 14.7 - 0.0125 = 14.6875, or 14.7 after rounding. This is our upper normal limit. So in our sample, the 95% normal range is 4.3 to 14.7. These confidence interval are easily calculated, however, appropriate normal reference groups may be difficult to identify.

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METHOD OF REPORTING RELATIVE ANTIBODY ACTIVITY BY EIA

Qualitative and Semi-Quantitative Reports

Qualitative reports (e.g., "Positive") and semi-quantitative reports (e.g., Positive +++) have the advantage that they allow visual reading of the test result and that the report is easily understood.

However, visual readings are subjective, rely heavily on the skill of the reader, and may be influenced by the relative frequency of positives and negatives. Even if results are read by photometry or fluorimetry, the qualitative and semi-quantitative reports fail to make full use of the quantitative capacity of EIA as an assay and are of limited use in sero-epidemiologic comparison of patient group or in following the time-course of infection.

Quantitative Reports

Titration Method

In the titration method, antisera under test are serially diluted to the point, or titer, at which the specific antibody activity can no longer be detected, hence this method gives a truly quantitative measure of the relative antibody activity and is the standard by which other quantitative methods will be assessed. Titers are commonly reported as serum dilutions and expressed as ratios (1:1024) or reciprocals (1024).

The advantages of this method are:

1. Titers are highly quantitative measure of relative antibody activity.
2. Visual readings can be used.
3. The concept is easily understood.

Disadvantages of the titration method are:

1. Titers can be inaccurate and variable because EIA dose-response curves flatten towards titer end-point.
2. Serial dilutions are required, thus technical errors are compounded and reproducibility is only ± 50% (i.e., one two-fold dilution).
3. Titration scales are not continuous, thus the patient is allocated to one of a limited range of discrete quantitative categories, usually less than 12 serial dilutions.
4. Titrations are relatively work-intensive and costly of materials and reagents.
5. The possibility of confusion exists for the clinician regarding the "significant titer" which varies greatly between assay systems (CF, IFA, IHA, etc.).

Absorbance Method

Workers using ELISA for specific antibody activity commonly express results in absorbance values, such as A_{405} nm, when chromagenic substrates are used or in arbitrary fluorescence units when using fluorogenic substrates. However, these values should be considered as raw laboratory data analogous to counts per minute in RIA, and should be further refined before communication to the clinician.

Advantages of reporting absorbance values are:

1. Tests a single serum dilution.
2. Requires no data processing.
3. A continuous numerical scale is used covering a broad range, thus patients can be categorized into many quantitative categories usually with a reproducibility of ± 10% Coefficient of Variation.

Disadvantages of reporting absorbance values are:

1. Absorbance values are not linearly proportional to titers such that differences between sera with high antibody activities are minimized. For example, a one log increase of titer is not reflected by a one log increase in absorbance (Figure 6).
2. Absorbance values are not readily comprehensible to clinicians.

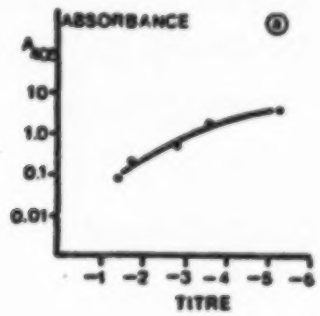

Figure 6. Relationship between absorbance values and titer.

Ratio Methods

Ratio methods include both Positive:Negative (P/N) ratio and the Percent Positive Method. While these two methods are very similar lets consider them separately.

Percent Positive Method

The patients result is expressed as a percentage of that simultaneously derived from the positive reference serum or pool. While P/N ratios of 2 or 3 are usually considered positive, no such constant feature applies to the Percent Positive method.

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Advantages of the percent positive method:

1. The ratio compares the patient to a positive reference in a quantitative fashion.
2. The results are easily understood.
3. Tests are performed on a single-serum dilution only.

Disadvantages of the percent positive method:

1. If the positive reference absorbance value is too high the percent positive decreases and becomes difficult to reproduce. A serum that falls somewhere around a moderate-strong positive would be well suited to be the positive reference serum.

Positive:Negative (P/N) Ratio

By this method, specific antibody level of patients serum is expressed as a percentage of that simultaneously derived from the negative reference serum or pool. P/N ratios of 2-3 are usually considered positive.

Advantages of P/N Ratio:

1. The ratio compares the patient's test value to the normal population (P/N) or positive reference in a quantitative fashion.
2. The result is easily understood.
3. Tests can be run on a single-serum dilution.

Disadvantages of P/N Ratio:

1. If the negative reference absorbance is too low, <0.10 for example, ratios increase greatly and become difficult to reproduce.
2. A disadvantage common to both the percent positive and P/N ratio methods is that neither are linearly proportional to titer (Figure 7).

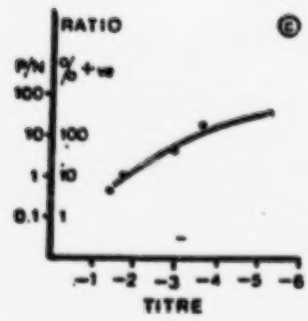

Figure 7. Relationship between ratio methods and serum titer.

Multiples of Normal Activity (MONA)

The Multiple of Normal Activity is a special case of the ratio method and represents a direct attempt to correct the deficiencies of reporting raw absorbance data from assays at single serum dilution. To use MONA, the EIA test system is optimized and the working dilution for test sera is determined.

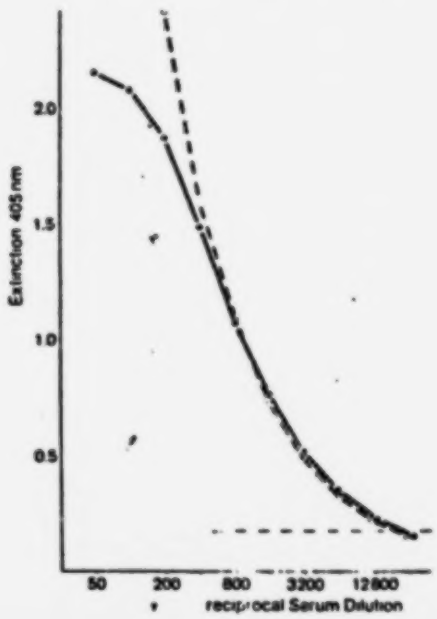

Figure 8. Titration of a serum Stick-ELISA from a patient with hydatid disease (O-------O) and parabolic approximation (●————●), calculated from extinctions of serum dilutions 1:800 and 1:25600. The broken horizontal line gives the extinction of a normal serum at working dilution.

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Here we have the actual dose-response curve represented (●———●). It is known by mathematical analysis that the true relationship between antibody titer and the response to changes in titer (Extinction) is parabolic. However, due to factors inherent to the test conditions and our inability to measure absorbance greater than 2.0, the curve will deviate from a true parabola at high antibody concentration. The true parabolic curve (---) can be described by computing the parabolic exponent (n):

$$n = \frac{\log DIL\text{-}F}{\log A_1 \log A_2}$$

DIL-F = dilution factor

A_1 = absorbance of the initial dilution of reference serum

A_2 = absorbance of a dilution of the positive reference serum

The parabolic exponent is derived from the absorbances (A) of a pathological standard serum and a dilution of it (A_1 and A_2). DIL-F is the dilution factor of the positive serum. The standard dilution should consist of several two-fold dilution steps to ensure prolonged approximation. The parabolic exponent (n) remains constant for the test system and requires re-calculation only if test conditions change; for example, a new conjugate is prepared. For use, all subsequent test absorbance values are transformed to multiples of normal activity (MONA) by relating the test absorbance (Ax) to that of the normal reference pool (A_{neg}), tested simultaneously, according to either equation [2] or [3]. These equations place the Ax value on the computed parabolic curve.

[2] $MONA = \text{antilog}\ (n\ (\log Ax - \log A_{neg}))$

[3] $MONA = \left(\frac{Ax}{A_{neg}}\right)^n$

Ax = test absorbance of unknown

A_{neg} = test absorbance of "normal" reference pool

n = parabolic exponent

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Advantages of MONA:

1. MONA compares the specific antibody activity of the patient to that of the "normal" population in a quantitative manner.
2. The results are more linearly proportional to titer due to the use of the parabolic exponent (Figure 9).
3. Requires the test of a single serum dilution only.

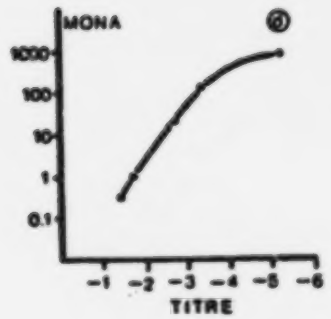

Figure 9. Relationship between MONA and serum titer.

Disadvantages of MONA:

1. Calculation of (n) is greatly influenced by the choice of positive reference serum used and the portion of the dose-response curve in the calculation.
2. MONA assumes dose-response curves of all test sera to be parallel.
3. Not valid for absorbance values above the parabolic approximation.

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Reference-Value/Percentile Method

In this method a large number (>120) of normal individual from an appropriate reference group is tested for specific antibody by ELISA under standard conditions and a frequency distribution of these reference absorbance values is prepared. Percentiles of reference population are determined from the cumulative percent of the population at each absorbance value and a standard curve of percentiles vs. absorbance value can be prepared. Absorbance values of test sera in subsequent tests can then be transformed to percentiles or P values from the standard curve. An example result would be expressed as:

Anti-X antibody activity ELISA value = 0.52 (A_{405}) p = 0.98

Reference Group: Rural Nigerian Adults; n = 900.

By comparing percentile markers, subtle seroepidemiological differences between specific groups can be detected.

Advantages of Reference-value/Percentile method:

1. Results can be expressed relating the patient's level of specific antibody to that found in a carefully selected and defined reference group.
2. Useful for seroepidemiologic comparison.

Disadvantages of Reference-value/Percentile method:

1. Requires initial testing of large numbers of reference group subjects.
2. Selection of the appropriate reference group can be difficult.
3. If the reference group is "normal,"" only negative patients are quantified while most positive values are reported as >100th percentile or P>1.0, thus no quantitative information is available on those patients for whom it is most needed.
4. Results are not linearly proportional to titer (Figure 10).

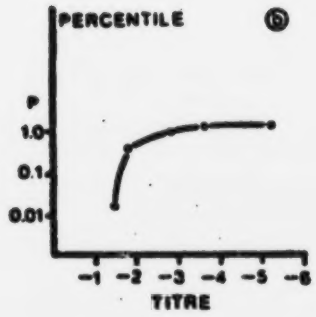

Figure 10. Relationship between Reference-value/Percentile method and serum titer.

Effective Dose Method

The Effective Dose Method proposed by Leinikki et al. (10) is a special case of the titration method whereby improved results from:

(a) determining the titer from the linear rather than the flattening phase of the dose-response and

(b) expressing the titer as the dilution difference (log distance) between the dose-response curves of the test and reference samples rather than as the dilution of the test serum itself.

An example report would be:

Acute Phase Serum : EIA ED = -0.96

Convalescent Phase Serum : EIA ED = +1.23

Units from Standard Curve

A standard curve can be prepared from computor assisted or manual analysis of routine serum titrations obtained under standard test conditions. At least 40 sera, representing the complete range of specific antibody activities from weak negative to strong positive are titrated in two-fold dilutions to their respective end-points. The dose-response curves of each serum are plotted on

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log paper and the titers taken at the serum dilution where the curve intersects with the positive-negative cut-off line (Figure 11).

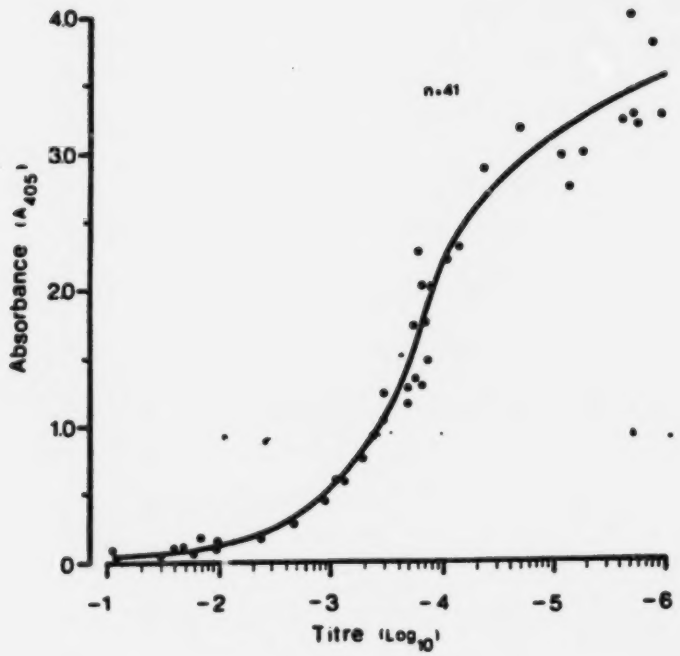

Figure 11. Standard curve method of expressing antibody concentration. Curve based upon 41 sera of different antibody concentrations.

A scatter plot is then prepared by plotting for each serum the absorbance value at the routine test working dilution versus its respective end-point. A sigmoidal plot will result with non-uniform variance (scatter) occurring at the higher and lower absorbance values.

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The usual curve-fitting technique and curvilinear regression with weighting to correct for the variance can be applied to produce a standard curve or equation which is used to transform EIA values obtained at the working dilution to titer, international units or other units. This step can be assisted by a programmable calculator linked to the photometer. With careful use of reference standard sera to maintain interassay quality control the same standard curve parameters can be used daily.

Advantages of the Dose-Response Curve Method:

1. Absorbance values are transformed to highly quantitative, easily understood units on a continuous scale.
2. Sera are tested at a single serum dilution.
3. Results are linearly proportional to titer.

Disadvantages of the Dose-Response Curve Method:

1. Parallel dose-response curves are assumed.
2. Rigorous quality control is required.
3. Not valid for high absorbance values.

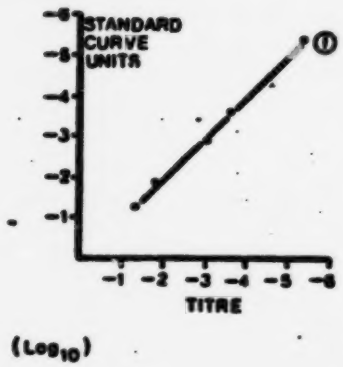

Figure 12. Standard curve vs. titer.

SUMMARY FOR

METHODS OF REPORTING RELATIVE ANTIBODY ACTIVITY

The transition of EIA from use in research applications to routine diagnostic applications necessitates the re-thinking of reporting methods to produce a form of EIA test results intelligible to clinicians. The quantitation of specific antibody activity by EIA poses special problems due to the large range of activities being measured, the multiplicity of affinities, the possibility of non-parallel dose-response curves, and to the competitive effects of specific responses in different immunological classes.

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In relation to a list of features characterizing the ideal serological report, no single method was found to satisfy all requirements. The Standard Curve method came closest to meeting the requirement and yet even it is not completely satisfactory. Further efforts will be needed to devise a means of transforming raw EIA data (absorbance units of fluorescence units) from assays at a single serum dilution to units on a continuous scale, linearly proportional to titer; the object being to provide the clinician with a reliable estimate of the relative antibody activity specific for the antigen in question.

This presentation was adapted from:

D. deSavigny and A. Voller. 1980. The communication of ELISA data from laboratory to clinician. J. Immunoassay 1:105-108.

Other References

1. de Savigny DH, Voller A, Woodruff AW. Toxocariasis: Diagnosis by Enzyme Immunoassay. J. Clin Path 1979; 32:284-88.

2. Ashton WD. The logit transformation with Special Reference to its Uses in Bioassay. Hainer Publishing Co. New York, 1972.

3. Marschner I, Erhart F, Scriba PC. In: Radioimmunoassay and Related Procedures in Medicine. Vol. I. I.A.E.A. Vienna, 1974; 111-22.

4. Rodbard D, Hutt DM, In: Radioimmunoassay and Related Procedures in Medicine. Vol. I. I.A.E.A. Vienna, 1974; 165-92.

5. Butler JE, Feldbush TL, McGivern PL, Stewart N. The enzyme-linked immunosorbent assay (ELISA): A measure of antibody concentration or affinity? Immunochemistry 1978; 15:131-36.

6. Mainland D. Remarks on clinical "norms". Clin. Chem. 1971; 17:267-74.

7. Reed AH, Henry RJ, Mason WB. Influence of statistical method used on the resulting estimate of normal range. Clin. Chem. 1971; 17:275-84.

8. McLaren M, Draper CC, Robert JM, Minter-Goedblood E, Lighthart GS, et al. Studies on the enzyme-linked immunosorbent assay (ELISA) test for Schistosoma mansoni infections. Annals Trop. Med. Parasit. 1978; 72:243-53.

9. Felgner P. Stepless antibody determination with the stick-ELISA technique. Results expressed as multiple of normal activity (MONA). Zbl. Bakt. Hyg. I. Abt. Orig. A. 1978; 242:100-05.

10. Leinikki PO, Passila S. Evaluation of some of the parameters of the enzyme-linked immunosorbent assay. J. Infect. Dis. 1977; 136 (Suppl.): S294-99.

11. Leinikki PO, Shekarchi I, Dorsett P, Sever JL. Enzyme-linked immunospecific assay determination of specific rubella antibody levels in micrograms of immunoglobulin G per milliliter of serum in clinical samples. J. Clin. Microbiol. 1978; 8:419-23.